fermentation

MDPI

Article

Dynamic Regulation of Transporter Expression to Increase L-Threonine Production Using L-Threonine Biosensors

Sumeng Wang, Ruxin Hao, Xin Jin, Xiaomeng Li, Qingsheng Qi * and Quanfeng Liang *

State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China; smwang001@126.com (S.W.); hrx970625@163.com (R.H.); jinx.in@outlook.com (X.J.); lleky0713@163.com (X.L.)
* Correspondence: addresses: qiqingsheng@sdu.edu.cn (Q.Q.); liangquanfeng@sdu.edu.cn (Q.L.)

Abstract: The cytotoxicity of overexpressed transporters limits their application in biochemical production. To overcome this problem, we developed a feedback circuit for L-threonine production that uses a biosensor to regulate transporter expression. First, we used IPTG-induced *rhtA* regulation, L-threonine exporter, to simulate dynamic regulation for improving L-threonine production, and the results show that it had significant advantages compared with the constitutive overexpression of *rhtA*. To further construct a feedback circuit for *rhtA* auto-regulation, three L-threonine sensing promoters, P_{cysJ}, P_{cysD}, and P_{cysJH}, were characterized with gradually decreasing strength. The dynamic expression of *rhtA* with a threonine-activated promoter considerably increased L-threonine production (21.19 g/L) beyond that attainable by the constitutive expression of *rhtA* (8.55 g/L). Finally, the autoregulation method was used in regulating *rhtB* and *rhtC* to improve L-threonine production and achieve a high titer of 26.78 g/L (a 161.01% increase), a yield of 0.627 g/g glucose, and a productivity of 0.743 g/L/h in shake-flask fermentation. This study analyzed in detail the influence of dynamic regulation and the constitutive expression of transporters on L-threonine production. For the first time, we confirmed that dynamically regulating transporter levels can efficiently promote L-threonine production by using the end-product biosensor.

Keywords: biosensor; dynamic regulation; exporter; L-threonine production

Citation: Wang, S.; Hao, R.; Jin, X.; Li, X.; Qi, Q.; Liang, Q. Dynamic Regulation of Transporter Expression to Increase L-Threonine Production Using L-Threonine Biosensors. *Fermentation* **2022**, *8*, 250. https://doi.org/10.3390/fermentation8060250

Academic Editors: Mohamed Koubaa and Xian Zhang

Received: 19 April 2022
Accepted: 24 May 2022
Published: 26 May 2022

1. Introduction

L-threonine (Thr, CAS 72-19-5), an essential amino acid for mammals, is widely used in food, cosmetics, animal feed, and pharmaceuticals [1]. L-threonine is one of four major commercial amino acids, the others being L-glutamic acid, L-lysine, and DL-methionine. The global production of L-threonine totals 700,000 metric tons annually [2]. Currently, microbial fermentation with catalyzation by six enzymes from oxalacetate—including *aspC*-encoded aspartate aminotransferase, *thrA* (*metL* or *lysC*)-encoded aspartate kinase and homoserine dehydrogenase, *asd*-encoded aspartyl semialdehyde dehydrogenase, *thrB*-encoded homoserine kinase, and *thrC*-encoded threonine synthase—is the main method of L-threonine production (Figure 1) [3]. L-threonine belongs to the aspartate family of amino acids. First, L-aspartate is generated from oxaloacetate by L-aspartate aminotransferase. L-aspartate is then catalyzed by aspartate kinase to aspartate phosphate. This is followed by a two-step reduction reaction catalyzed by aspartyl semialdehyde dehydrogenase and homoserine dehydrogenase to generate L-homoserine. The homoserine kinase catalyzes the phosphorylation of L-homoserine to L-homoserine phosphate, which is finally catalyzed by threonine synthase to L-threonine.

Many strategies have been explored to synthesize L-threonine, including cofactor engineering, energy adjustment, the overexpression of rate-limiting steps, the removal of feedback inhibition, and the removal of competitive pathways [1,3–5]. Transporter engineering has also emerged as an attractive approach to improve L-threonine production [6–10]. The overexpression of transporters is a commonly used tactic to increase strain

tolerance by pumping out products [3,7,8,11]. However, the overexpression of membrane proteins seriously hinders the homeostasis of proteins in the cytoplasm [12,13], which is not conducive to efficient catalytic anabolites. For example, overexpressing membrane proteins can reduce the levels of the respiratory chain complexes succinate dehydrogenase and cytochrome *bd* and bo_3 oxidases in *Escherichia coli* [13]. Therefore, the expression of transporters should be carefully modified for chemical production without disturbing the physiological metabolism. In addition, some transporter engineering strategies were also performed in microbial manufacturing, such as omics-guided transporter mining, transporter evolution to enhance transport efficiency and substrate specificity, and the heterologous expression of transporter [9]. However, transporters obtained from the above strategies still need to be overexpressed to improve product outflow. One promising method of performing this is to dynamically regulate transporter expression through a general biosensor. Dynamic regulation can automatically control the level of transporters according to the needs of cells, which can not only alleviate the adverse effects of overexpressed transporters on cells, but also timely discharge intracellular metabolites. To produce n-butanol and 1-alkene, previous studies have induced transporter expression by responding to membrane stress and exhausted glucose [14,15]. However, such a general biosensor needs to be coupled with the cell growth state to perform its regulatory functions. The cell growth state can be affected by many factors, such as hyperosmotic stress condition [16], which lead to the non-precise regulation of transporter expression by using the cell-growth-state-dependent biosensor. In contrast to the general biosensor, a product-specific biosensor finely regulates transporter expression without being affected by culture conditions and changes in cell physiology. Many end-product-specific biosensors have been designed and utilized to dynamically regulate metabolic pathways to improve chemical production [17,18]. Although Lange et al. found that the exporter BrnFE can be activated by the transcription factor *lrp* and that its expression increased with the addition of branched-chain amino acids [19], the effects of dynamically regulated transporter expression by using end-product biosensors have not been studied in detail. The comparison of the effects of dynamic regulation and the constitutive expression of transporters on product synthesis has not been reported.

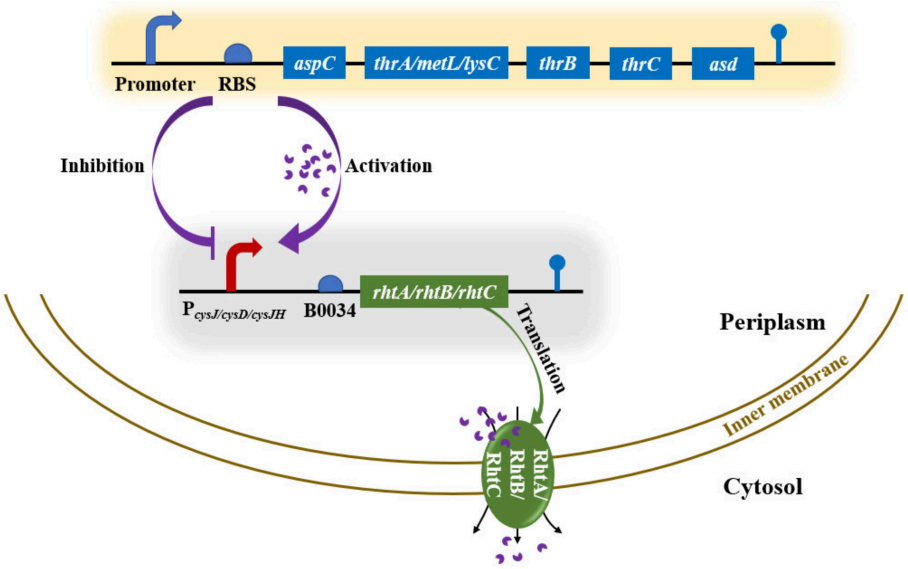

Figure 1. Scheme of sensing L-threonine to regulate transporter expression. The L-threonine production pathway consists of five genes: aspartate aminotransferase encoded by *aspC*; aspartate kinases encoded by *thrA*, *metL*, or *lysC*; semialdehyde dehydrogenase encoded by *asd*; homoserine dehydrogenase encoded by *thrA*; homoserine kinase encoded by *thrB*; and threonine synthase encoded by *thrC*. Three transporters were encoded by *rhtA*, *rhtB, and rhtC*. L-threonine transporters belong to the inner membrane. P_{cysJ}, P_{cysD}, and P_{cysJH} are L-threonine-sensed promoters for activating gene translation.

In this study, we use the native L-threonine-sensing promoters P_{cysJ}, P_{cysD}, and P_{cysJH} to study in detail the effect of dynamically regulated transporter expression for L-threonine production (Figure 1). Compared with the constitutive expression of transporters, dynamic regulation significantly improved L-threonine production with an increase of approximately 147%. This regulation can also be extended to two other native transporters, *rhtB* and *rhtC*.

2. Materials and Methods

2.1. Chemicals and Reagents

The L-threonine standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Phanta HS Super-Fidelity DNA Polymerase was purchased from Vazyme Biotech (Nanjing, China). High-performance liquid chromatography grade acetonitrile (75-05-8) was obtained from Tedia (ACN, Tedia Company, Inc., Fairfield, OH, USA). Isopropyl-β-d-thiogalactoside (IPTG; CAS, 367-93-1) and spectinomycin dihydrochloride pentahydrate (CAS, 22189-32-8) were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. Triethylamine (CAS, 121-44-8) and phenyl isothiocyanate (CAS, 103-72-0) were purchased from Aladdine. Other reagents were purchased from Sinopharm Chemical Reagent Beijing Co. Ltd. (Shanghai, China).

2.2. Construction of Strains and Plasmids

All strains and plasmids used in this study are listed in Tables S1 and S2. *E. coli* DH5α was used to reconstruct plasmids, and *E. coli* K-12 MG1655 was used for L-threonine sensor characterization. The L-threonine-producing Tm strain was provided by Fufeng Group. The strains Tm-100AR, Tm-101AR, and Tm-109AR containing plasmids cl-100AR, cl-101AR, and cl-109AR, respectively, were constructed to study the effect of *rhtA* levels on L-threonine production. We introduced pCL1920 and cl-*rhtA* into Tm to construct strains Tm-cl and Tm-AR to study the effects of *rhtA* expression times on cell growth and L-threonine production. MG-cl, MG-P_{cysJ}, MG-P_{cysD}, and MG-P_{cysJH} were constructed by transforming the plasmids pCL1920, cl-JAR, cl-DAR, and cl-JHAR into *E. coli* K-12 MG1655. Tm-P_{cysJ}, Tm-P_{cysD}, and Tm-P_{cysJH} were constructed by transforming the plasmids cl-JAR, cl-DAR, and cl-JHAR into Tm. Tm-JAR, Tm-DAR, Tm-JHAR, Tm-105AR, Tm-110AR, and Tm-116AR were engineered by introducing cl-JAR, cl-DAR, cl-JHAR, cl-105AR, cl-110AR, and cl-116AR into Tm to analyze the influence of the dynamic regulation of *rhtA* on L-threonine production. Tm-JB and Tm-JC were constructed by introducing cl-JB or cl-JC into Tm and used to further dynamically regulate the native transporters to improve L-threonine production. All engineered strains were constructed by transforming the corresponding plasmids into *E. coli* K-12 MG1655 or Tm with the calcium chloride ($CaCl_2$) or electroporation transformation methods.

The primers used for plasmid reconstruction are listed in Table S3. The low copy number plasmid pCL1920 with the pSC101 replicon was used as the cloning vector. All plasmids in this study were constructed using the Gibson assembly method [20]. To construct cl-*rhtA*, the backbone was amplified from pCL1920 with the primers cl-F1 and cl-R1, and *rhtA* was amplified from the *E. coli* K-12 MG1655 genome with the primers cl-rhtA-F and cl-rhtA-R by using Phanta HS Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing, China). cl-*rhtA* was assembled by fusing the amplified backbone and *rhtA* with the 2X MultiF Seamless Assembly Mix (ABclonal Technology Co., Ltd. Wuhan, China). P*cysJ-rfp*, P*cysD-rfp*, and P*cysJH-rfp* were constructed through fusing the promoters P_{cysJ}, P_{cysD}, and P_{cysJH} amplified from the *E. coli* K-12 MG1655 genome with the reporter *rfp* and then was inserted into amplified pCL1920 backbone. The dynamically regulated *rhtA* plasmids containing cl-JAR, cl-DAR, and cl-JHAR were constructed with the frame of the $P_{cysJ/cysD/cysJH}$-B0034-*rhtA*-terminator BBa_B1006-P_{cysJ} B0034-*rfp*. To constitutively express *rhtA*, we selected three different transcription levels of promoters (J23105, J23110, and J23116) from iGEM (http://parts.igem.org/Main_Page /(accessed on December 2021)). cl-105AR, cl-110AR, and cl-116AR were constructed according to the form of the $P_{J23105/J23110/J23116}$-B0034-*rhtA*-terminator BBa_B1006-P_{cysJ} B0034-*rfp*. The native L-threonine exporters *rhtB* and *rhtC*

were amplified from the *E. coli* K-12 MG1655 genome and cloned into pCL1920 to generate the recombinant plasmids cl-JB and cl-JC for dynamic regulation.

2.3. Characterization of the L-Threonine-Sensing Promoters

To characterize the promoters P_{cysJ}, P_{cysD}, and P_{cysJH}, the fluorescence intensity of the reporter gene *rfp* was monitored using a multidetection microplate reader (Synergy HT, BioTek, Winooski, VT, USA). Single colonies were grown in 12-well microassay plates with 2 mL of LB medium supplemented with spectinomycin dihydrochloride pentahydrate at 37 °C for approximately 12 h. The feeds were then transferred to a 96-well microassay plate containing 200 µL Luria–Bertani (LB) medium with 2% (*v*/*v*) inoculation at 37 °C for 48 h. Spectinomycin dihydrochloride pentahydrate was added during the cultivation process to maintain the stabilization of the plasmids. The fluorescence intensity of *rfp* was measured through excitation at 590 nm and emission at 645 nm. The characterization was calculated with the ratio of the fluorescence intensity of *rfp* to the optical density at 600 nm (OD_{600}). All measurements were performed in triplicate.

2.4. Culture Medium and L-Threonine Fermentation

A LB medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl was used for plasmid construction and L-threonine sensor characterization. The L-threonine fermentation medium consisted of 15 g/L of $(NH_4)_2SO_4$, 2 g/L of KH_2PO_4, 1 g/L of $MgSO_4 \cdot 7H_2O$, 2 g/L of yeast extract, and 0.02 g/L of $FeSO_4$ [8]. We also added 40 g/L of glucose as the initial carbon source and 20 g/L $CaCO_3$ to adjust the pH during fermentation. For shake-flask fermentation, single colonies were cultured in fresh LB medium at 37 °C for approximately 12 h. The precultured seeds were subsequently transformed into a 300 mL shake flask attached with flaps containing a 20 mL fermentation medium with 1% (*v*/*v*) inoculation. Fermentation was performed at 220 rpm, 37 °C for 36 h. When glucose was lower than 10 g/L, it was supplemented up to 40 g/L. *rhtA* expression was induced with 0.1/0.2/0.5 mM of IPTG when necessary.

Analytical Methods

A 1 mL culture was vigorously vortexed, and 0.1 mL of it was transferred to a 1.5 mL centrifuge tube. We added 0.9 mL of 1 mM HCl and mixed the solution to remove the residual $CaCO_3$. Subsequently, OD_{600} was measured using a spectrophotometer (Shimazu, Kyoto, Japan). For glucose and L-threonine detection, the culture was centrifuged at 12,000 rpm for 2 min to collect the supernatant. The collected supernatant was filtered with a 0.22 µm aqueous membrane for glucose analysis. Glucose was quantified with a high-performance liquid chromatography (HPLC) system (Shimadzu, Kyoto, Japan) equipped with a refractive index detector (RID-10A; Shimadzu, Kyoto, Japan) and an Aminex HPX-87H ion exclusion column (Bio-Rad Laboratories, Hercules, CA, USA); 5 mM H_2SO_4 was used as the mobile phase at a flow rate of 0.6 mL/min [21].

For the detection of L-threonine, the collected supernatant was initially deproteinized with 5% trichloroacetic acid. Subsequently, the pretreated supernatant was derived with triethylamine and phenyl isothiocyanate and then extracted with n-hexane. Briefly, 0.2 mL of samples and standard L-threonine were pretreated with the mixture of triethylamine–acetonitrile (1.4 mL of triethylamine mixed with 8.6 mL of acetonitrile). Next, we added phenyl isothiocyanate–acetonitrile (25 µL of phenyl isothiocyanate mixed with 2 mL of acetonitrile) to pretreat the samples and standard L-threonine for 1 h at room temperature. We added 0.4 mL of n-hexane and vigorously shocked. The bottomed liquid (0.2 mL) was collected and diluted with 0.8 mL of deionized water. The solution was filtered with a 0.22 µm organic membrane, and samples were detected using an HPLC equipped with a diode array detector (SPD-M20A; Shimadzu, Kyoto, Japan), with the VenusilAA (4.6 × 250 mm, 5 µm, Agela Techanologies) column applied at 40 °C. The analysis was performed at a flow rate of 1 mL/min with mobile phases consisting of (A) 15.2 g of sodium acetate dissolved in 1850 mL of ultrapure water and mixed with 140 mL of acetonitrile and

(B) 80% (*v*/*v*) acetonitrile and 20% (*v*/*v*) ultrapure water. The concentration of L-threonine was quantified with the peak area according to the corresponding standard curve.

2.5. Statistical Analysis

Results are presented as the mean ± SEM. Differences between the means were evaluated using a one-way ANOVA. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Simulating Dynamic rhtA Regulation with IPTG Induction

The membrane protein RhtA consists of 10 predicted transmembrane segments, which were identified and characterized as L-threonine and L-homoserine transporters [22]. Constitutive overexpressing *rhtA* is the commonly method used to improve L-threonine production and removing the toxic intermediate metabolite L-homoserine [22–26]. In this study, we used the industrial L-threonine production host Tm, engineered from *E. coli* K-12 MG1655, from the Fufeng Group (http://www.fufeng-group.com/ (accessed on January 2021)) as the parent strain.

To verify whether the dynamic regulation of transporter expression can improve L-threonine production, we firstly explored the effect of constitutive overexpressed *rhtA* on L-threonine production. Three promoters at strong (J23100, 1348), medium (J23101, 399), and weak (J23109, 18) from the Registry of Standard Biological Parts (http://parts.igem.org/Main_Page(accessed on January 2021)) were characterized in strains Tm-100AR, Tm-101AR, Tm-109AR, respectively (Figure 2A). As illustrated in Figure 2A, compared with the control (strain Tm-cl without *rhtA* overexpression), overexpressing *rhtA* enhanced L-threonine production due to relief from the toxicity of the accumulated products through the timely export of L-threonine [9,27]. Tm-100AR and Tm-109AR significantly increased the titer of L-threonine ($p < 0.0001$) than Tm-101AR. However, there was an irregular correlation between the increase in production and the intensity of *rhtA* levels. Then, we used IPTG-induced *rhtA* regulation to simulate the availability of dynamic regulation. *rhtA* was induced with 0.5 mM of IPTG in different growth phases (OD 0, 3, 6, or 9) (Figure 2B). When enhancing *rhtA* expression at different growth stages, host cells with delayed *rhtA* induction achieved a higher L-threonine titer than constitutive expression (Figure 2B). *rhtA* expressed at OD 3 achieved higher titers at 15.4 g/L than early at OD 0 ($p < 0.05$) with 13.4 g/L or delayed induction at OD 6 ($p < 0.01$) or 9 ($p < 0.001$) with 13.4 g/L and 10.7 g/L, respectively. The premature expression of high-level *rhtA* causes membrane burden. *rhtA* expresses too late to excrete the accumulated L-threonine with low-level *rhtA* in the cell timely, resulting in metabolic burden. Compared to the strength of the constitutive promoter shown in Figure 2A, the promoter strength of Plac-lacO (RFP/OD_{600} values from 6.7 to 55.4) can be evaluated to be roughly between J23101 (RFP/OD_{600} value, 399) and J23109 (RFP/OD_{600} value, 18), suggesting that increased L-threonine productivity at OD 0–9 may be not due to higher intensity of Plac-lacO. Therefore, the dynamic regulation of *rhtA* in different fermentation phases can improve L-threonine production, and it had significant advantages compared with the constitutive overexpression of *rhtA*. Although the transporter *rhtA* was successfully regulated to increase L-threonine production, the addition of the exogenous inducer IPTG is impracticable in industrial production due to the high production cost [28].

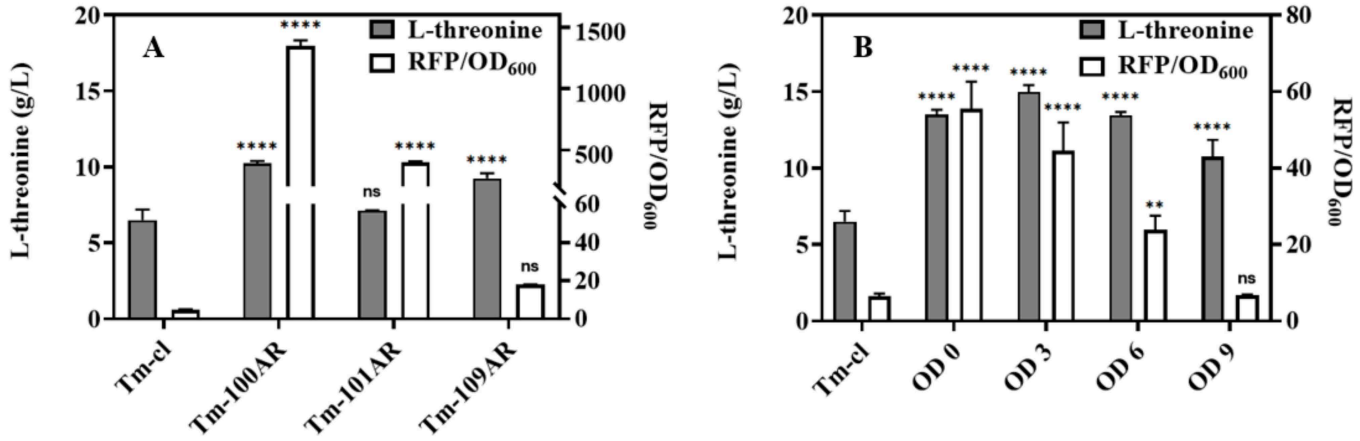

Figure 2. Adding IPTG at different growth stages to dynamically control *rhtA* expression. (**A**) *rhtA* was constitutively expressed with strong (promoter J23100), medium (promoter J23101), and weak (promoter J23109) intensities in strains Tm-100AR, Tm-101AR, and Tm-109AR, respectively. Strain Tm-cl without overexpressed *rhtA* as the control. (**B**) The Tm-AR strain containing overexpressed *rhtA* was induced with 0.5 mM of IPTG at OD 0, 3, 6, and 9. L-threonine titers were measured at 36 h of fermentation. All results were calculated with three (n = 3) independent replicates. Error bars represent mean ± SEM. **** $p < 0.0001$, ** $p < 0.01$. ns: non-significance.

3.2. Characterizing the L-Threonine-Sensing Promoters

Biosensors have emerged as an outstanding tool for dynamically adjusting gene expression in metabolic engineering [18,29]. Several *E. coli*-derived L-threonine-sensing promoters have been excavated through omics analysis [30]. In this paper, the L-threonine sensor was characterized by placing the report gene *rfp* under the promoter P_{cysJ}, P_{cysD}, or P_{cysJH}. We initially tested the response in *E. coli* K-12 MG1655 with the strains MG-P_{cysJ}, MG-P_{cysD}, and MG-P_{cysJH}. After removing the background value of RFP/OD_{600} from strain MG-cl, MG1655 with empty plasmid pCL1920, all three promoters can be activated by adding L-threonine (Figure 3A). The intensity of expression continuously decreased for P_{cysJ}, P_{cysD}, and P_{cysJH}. To demonstrate that the three promoters were practicable in the L-threonine-producing strain and determine the lowest activation threshold, the strains Tm-P_{cysJ}, Tm-P_{cysD}, and Tm-P_{cysJH} were constructed by introducing the plasmid P_{cysJ}-*rfp*, P_{cysD}-*rfp*, or P_{cysJH}-*rfp* into the industrial strain Tm. The results demonstrate that the three promoters can be used to activate gene expression during the L-threonine production process (Figure 3B). Furthermore, expression levels increased with increased L-threonine concentration (Figure 3B,C). As indicated in Table S4, the three promoters had different minimum sensing thresholds during fermentation: P_{cysJ} and P_{cysJH} at 3 h with 0.05 g/L and P_{cysD} at 5 h with 0.25 g/L of L-threonine. The highest concentrations of L-threonine sensing were not measured because the fluorescence intensity of *rfp* continued to increase at the final concentration of L-threonine (Figure 3C). Nonetheless, we quantified the highest sensing threshold (~60 g/L) with MG-P_{cysJ}, MG-P_{cysD}, and MG-P_{cysJH} by exogenously adding different concentrations of L-threonine (Figure 3A).

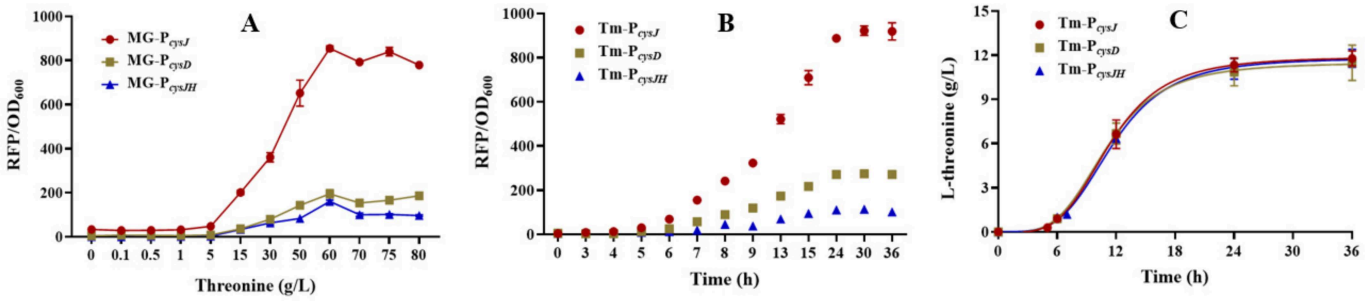

Figure 3. Characterization of the L-threonine-sensing promoters P$_{cysJ}$, $_{cysD}$, and $_{cysJH}$. (**A**) Plasmids P$_{cysJ}$-*rfp*, P$_{cysD}$-*rfp*, and P$_{cysJH}$-*rfp* were introduced into *E. coli* K-12 MG1655 to construct MG-P$_{cysJ}$, MG-P$_{cysD}$, and MG-P$_{cysJH}$ for characterization by adding 0–80 g/L L-threonine. Strain MG-cl containing the empty plasmid pCL1920 was the control for removing the background value of RFP/OD$_{600}$. (**B**) The sensing curves of the three promoters were monitored in the strains Tm-J, Tm-D, and Tm-JH containing the plasmids P$_{cysJ}$-*rfp*, P$_{cysD}$-*rfp*, and P$_{cysJH}$-*rfp*. (**C**) Monitoring of the L-threonine concentrations of Tm-J, Tm-D, and Tm-JH during the fermentation process. All results were calculated with three (n = 3) independent replicates.

3.3. Improving L-Threonine Production through the Dynamic Regulation of rhtA Expression

The characterized sensing promoters P$_{cysJ}$, P$_{cysD}$, and P$_{cysJH}$ were used for gradually increasing *rhtA* expression in the L-threonine-producing strain Tm, corresponding to the strains Tm-JAR, Tm-DAR, and Tm-JHAR, respectively. To estimate the expression of *rhtA*, the linked *rfp* gene was activated by L-threonine-responsive promoters. Varying strengths of promoters J23105, J23110, and J23116 were selected to constitutively express *rhtA* in the strains Tm-105AR, Tm-110AR, and Tm-116AR, respectively. The strain Tm-cl contained the empty vector as the control. The expression levels of *rhtA* in strains with L-threonine-responsive promoters were gradually increased during the fermentation process with the analysis of RFP/OD$_{600}$ (Figure 4A). As illustrated in Figure 4A, the promoter J23110 had the same effect as P$_{cysJ}$ on *rhtA* expression at 36 h. Comparing the constitutive expression of *rhtA* with the promoter J23100 in the strain Tm-110AR, dynamically regulating the strain Tm-JAR with P$_{cysJ}$ resulted in a significantly improved L-threonine production of up to 21.19 g/L of titers, an increase of approximately 147% than the unexpressed strain (Figure 4B). The other L-threonine-sensing promoters P$_{cysD}$ and P$_{cysJH}$ had lower L-threonine titers than P$_{cysJ}$, 19.11 and 9.49 g/L, respectively, which are a result of the weaker expression of *rhtA*. These results suggest that stronger dynamic expression of *rhtA* is more conducive to L-threonine production. The difference in expression levels of *rhtA* may be due to the different transcription and translation strengths of P$_{cysJ}$, P$_{cysD}$ and P$_{cysJH}$. It may also be caused by the different affinity for L-threonine of the currently unknown transcription regulators. Although a stronger constitutive expression of *rhtA* with the promoter J23116 can increase the L-threonine titer, it is still lower than the dynamic expression with the promoter P$_{cysJ}$ and with a slower cell growth (Figure 4C). These results indicate that the dynamic regulation of *rhtA* expression is more conducive to promoting L-threonine production. Dynamic regulation is a successful strategy to improve biochemical production [4,31–34]. Recently, a study has developed a thermal switch system to increase L-threonine yield through regulating carbon distribution and cofactor supplementation by sensing temperature [4]. Apart from the generally utilized metabolic flux engineering, in this study, we confirmed in the first time that dynamically engineering the transporter *rhtA* is more effective than constitutive expression for improving L-threonine production.

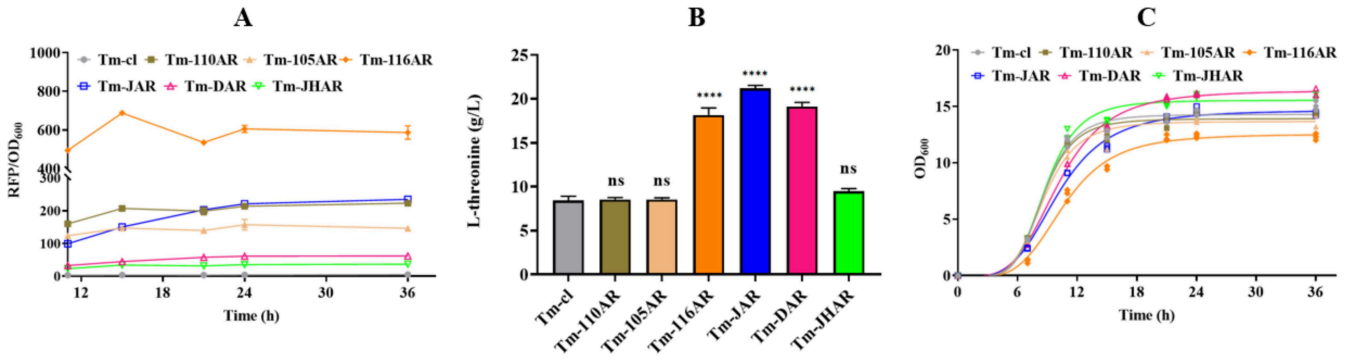

Figure 4. Analysis of the effects of dynamically and constitutively expressed *rhtA* on L-threonine production. (**A**) Analysis of the expression levels of *rhtA* during the whole fermentation process. Characterization of *rhtA* expression levels of the constitutive promoters (J23110, J23105, and J23116) and L-threonine-activated promoters (P_{cysJ}, P_{cysD}, and P_{cysJH}) with fluorescence intensity to OD_{600}. (**B**) L-threonine fermentation with dynamically or constitutively expressed *rhtA*. The constitutive promoters J23105, J23110, and J23116 were used to express *rhtA* in the strains Tm-105AR, Tm-110AR, and Tm-116AR, respectively. The L-threonine sensing promoters P_{cysJ}, P_{cysD}, and P_{cysJH} were used to control *rhtA* expression in the strains Tm-JAR, Tm-DAR, and Tm-JHAR, respectively. (**C**) Comparison of cell growth on varying strains with L-threonine-sensing promoters or constitutive promoters. All results were calculated with three (n = 3) independent replicates. Error bars represent mean ± SEM. **** $p < 0.0001$. ns: non-significance.

3.4. Regulating rhtB and rhtC to Further Improve L-Threonine Production

In *E. coli*, three native permeases—RhtA, RhtB, and RhtC encoded with *rhtA*, *rhtB*, and *rhtC*, respectively—were confirmed as the membrane proteins to pump out L-threonine [3]. RhtA belong to the category of nonspecific transporters for exporting L-threonine and L-homoserine. RhtB also transports homoserine lactone (HSL) [7,35]. By contrast, RhtC is the specific transporter for L-threonine [7]. Studies have reported that the expression of all these exporters results in the efficient pumping of L-threonine and increased production [1,3,36]. In this study, we demonstrated that L-threonine titers were considerably increased through the dynamic regulation of *rhtA* expression (Figure 4B). The sensing promoter P_{cysJ} had the greatest impact on increasing L-threonine production. To explore the influence of the autoregulated expression of *rhtB* and *rhtC* on L-threonine production, *rhtB* and *rhtC* were controlled by P_{cysJ} by responding to L-threonine (Figure 5). The plasmids P_{cysJ}-*rhtB* and P_{cysJ}-*rhtC* were transformed into the parent strain Tm and formed Tm-JB or Tm-JC. As illustrated in Figure 5A and Table 1, compared with the unexpressed transporter and overexpressed *rhtC*, the dynamically expressed *rhtB* resulted in the highest L-threonine titers of 26.78 g/L, an approximately 161% increase, and a productivity up to 0.734 g/L/h. Because Tm-JB consumed more glucose during the 36 h fermentation (Figure 5C), the yield (0.627 g/g) was slightly lower than that of Tm-JC (0.665 g/g). Although Tm-JB grew less than Tm-JC during the 0 to 18 h period, the final biomass at 36 h was higher (Figure 5B). These results demonstrate that dynamically regulating *rhtB* expression is relatively beneficial for L-threonine production.

Table 1. Titers, titer increase, yield, and productivity of L-threonine under dynamically regulated RhtB and RhtC.

	Titer (g/L)	Titer Increase (%)	Yield (g/g)	Productivity (g/L/h)
Tm-cl	10.26 (±0.68)	——	0.402 (±0.006)	0.285 (±0.019)
Tm-JB	26.78 (±0.67)	161.01 (±6.61)	0.627 (±0.023)	0.743 (±0.018)
Tm-JC	25.44 (±0.31)	147.95 (±3.02)	0.665 (±0.010)	0.706 (±0.008)

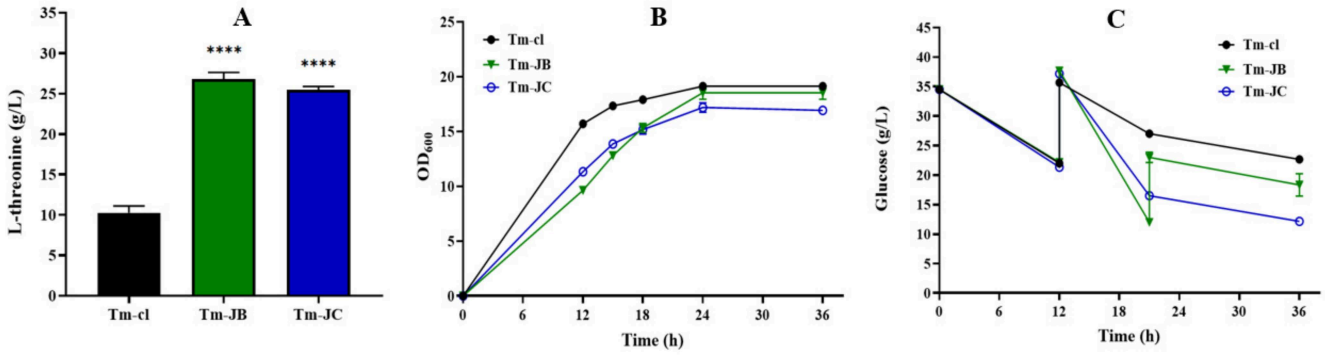

Figure 5. Dynamic regulation of *rhtB* and *rhtC* expression to improve L-threonine production. Strain Tm-cl containing the nonexpressed transporter as the control, *rhtB*, and *rhtC* was overexpressed in the strains Tm-JB and Tm-JC, respectively. (**A**) L-threonine titers were detected at 36 h of fermentation. (**B**,**C**) Glucose consumption and cell growth were monitored for 36 h of fermentation. All results were calculated with three (n = 3) independent replicates. Error bars represent mean ± SEM. **** $p < 0.0001$.

4. Conclusions

The toxicity of transporter expression to cells limits the application of transporter engineering in improving metabolite production. For the first time, we compared the effects of dynamic and constitutive expression transporters on product synthesis. We analyzed the effect of the dynamic regulation of transporters on L-threonine production based on end-product biosensors. These results demonstrate that the dynamic regulation of transporter expression was more conducive than constituent expression for L-threonine production, which increased by 147%. Therefore, the dynamic regulation of transporters is a promising strategy for improving biochemical production.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8060250/s1, Table S1. Plasmids used in this study; Table S2. Strains used in this study; Table S3. Primers used in this study; Table S4. Analysis of the initial sensing times and L-threonine concentrations of the three promoters in the L-threonine-producing strains [37].

Author Contributions: S.W.: Investigation, conceptualization, and writing original draft. R.H., X.J. and X.L.: Data curation and formal analysis. Q.Q. and Q.L.: Conceptualization, funding acquisition, resource management, project administration, supervision, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Key Research and Development Program of China (2019YFA0706900) and National Natural Science Foundation of China (31961133014, 31770095, 31971336).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Strain Tm was kindly provided by the Fufeng Group. We thank Chengjia Zhang and Nannan Dong from the Analysis and Testing Center of the State Key Laboratory for Microbial Technology (Shandong University) for assistance in the fermentation experiment.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Du, H.; Zhao, Y.; Wu, F.; Ouyang, P.; Chen, J.; Jiang, X.; Ye, J.; Chen, G.Q. Engineering Halomonas bluephagenesis for L-Threonine production. *Metab. Eng.* **2020**, *60*, 119–127. [CrossRef] [PubMed]
2. Wendisch, V.F. Metabolic engineering advances and prospects for amino acid production. *Metab. Eng.* **2020**, *58*, 17–34. [CrossRef] [PubMed]

3. Dong, X.; Quinn, P.J.; Wang, X. Metabolic engineering of Escherichia coli and Corynebacterium glutamicum for the production of L-threonine. *Biotechnol. Adv.* **2011**, *29*, 11–23. [CrossRef] [PubMed]
4. Fang, Y.; Wang, J.; Ma, W.; Yang, J.; Zhang, H.; Zhao, L.; Chen, S.; Zhang, S.; Hu, X.; Li, Y.; et al. Rebalancing microbial carbon distribution for L-threonine maximization using a thermal switch system. *Metab. Eng.* **2020**, *61*, 33–46. [CrossRef]
5. Lee, K.H.; Park, J.H.; Kim, T.Y.; Kim, H.U.; Lee, S.Y. Systems metabolic engineering of Escherichia coli for L-threonine production. *Mol. Syst. Biol.* **2007**, *3*, 149. [CrossRef]
6. Zhao, L.; Lu, Y.; Yang, J.; Fang, Y.; Zhu, L.; Ding, Z.; Wang, C.; Ma, W.; Hu, X.; Wang, X. Expression regulation of multiple key genes to improve L-threonine in Escherichia coli. *Microb. Cell Factories* **2020**, *19*, 46. [CrossRef]
7. Diesveld, R.; Tietze, N.; Fürst, O.; Reth, A.; Bathe, B.; Sahm, H.; Eggeling, L. Activity of exporters of Escherichia coli in Corynebacterium glutamicum, and their use to increase L-threonine production. *J. Mol. Microbiol. Biotechnol.* **2009**, *16*, 198–207. [CrossRef]
8. Kruse, D.; Krämer, R.; Eggeling, L.; Rieping, M.; Pfefferle, W.; Tchieu, J.H.; Chung, Y.J.; Saier, M.H., Jr.; Burkovski, A. Influence of threonine exporters on threonine production in Escherichia coli. *Appl. Microbiol. Biotechnol.* **2002**, *59*, 205–210.
9. Zhu, Y.; Zhou, C.; Wang, Y.; Li, C. Transporter Engineering for Microbial Manufacturing. *Biotechnol. J.* **2020**, *15*, e1900494. [CrossRef]
10. van der Hoek, S.A.; Borodina, I. Transporter engineering in microbial cell factories: The ins, the outs, and the in-betweens. *Curr. Opin. Biotechnol.* **2020**, *66*, 186–194. [CrossRef]
11. Pereira, R.; Wei, Y.; Mohamed, E.; Radi, M.; Malina, C.; Herrgård, M.J.; Feist, A.M.; Nielsen, J.; Chen, Y. Adaptive laboratory evolution of tolerance to dicarboxylic acids in Saccharomyces cerevisiae. *Metab. Eng.* **2019**, *56*, 130–141. [CrossRef]
12. Gubellini, F.; Verdon, G.; Karpowich, N.K.; Luff, J.D.; Boël, G.; Gauthier, N.; Handelman, S.K.; Ades, S.E.; Hunt, J.F. Physiological response to membrane protein overexpression in E. coli. *Mol. Cell. Proteom. MCP* **2011**, *10*, M111.007930. [CrossRef]
13. Wagner, S.; Baars, L.; Ytterberg, A.J.; Klussmeier, A.; Wagner, C.S.; Nord, O.; Nygren, P.A.; van Wijk, K.J.; de Gier, J.W. Consequences of membrane protein overexpression in Escherichia coli. *Mol. Cell. Proteom. MCP* **2007**, *6*, 1527–1550. [CrossRef]
14. Boyarskiy, S.; Davis López, S.; Kong, N.; Tullman-Ercek, D. Transcriptional feedback regulation of efflux protein expression for increased tolerance to and production of n-butanol. *Metab. Eng.* **2016**, *33*, 130–137. [CrossRef]
15. Zhou, Y.J.; Hu, Y.; Zhu, Z.; Siewers, V.; Nielsen, J. Engineering 1-Alkene Biosynthesis and Secretion by Dynamic Regulation in Yeast. *ACS Synth. Biol.* **2018**, *7*, 584–590. [CrossRef]
16. Su, Y.; Guo, Q.Q.; Wang, S.; Zhang, X.; Wang, J. Effects of betaine supplementation on L-threonine fed-batch fermentation by Escherichia coli. *Bioprocess Biosyst. Eng.* **2018**, *41*, 1509–1518. [CrossRef]
17. Xu, P.; Li, L.; Zhang, F.; Stephanopoulos, G.; Koffas, M. Improving fatty acids production by engineering dynamic pathway regulation and metabolic control. *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 11299–11304. [CrossRef]
18. Hossain, G.S.; Saini, M.; Miyake, R.; Ling, H.; Chang, M.W. Genetic Biosensor Design for Natural Product Biosynthesis in Microorganisms. *Trends Biotechnol.* **2020**, *38*, 797–810. [CrossRef]
19. Lange, C.; Mustafi, N.; Frunzke, J.; Kennerknecht, N.; Wessel, M.; Bott, M.; Wendisch, V.F. Lrp of Corynebacterium glutamicum controls expression of the brnFE operon encoding the export system for L-methionine and branched-chain amino acids. *J. Biotechnol.* **2012**, *158*, 231–241. [CrossRef]
20. Gibson, D.G.; Young, L.; Chuang, R.Y.; Venter, J.C.; Hutchison, C.A., 3rd; Smith, H.O. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **2009**, *6*, 343–345. [CrossRef]
21. Li, Y.; Li, M.; Zhang, X.; Yang, P.; Liang, Q.; Qi, Q. A novel whole-phase succinate fermentation strategy with high volumetric productivity in engineered Escherichia coli. *Bioresour. Technol.* **2013**, *149*, 333–340. [CrossRef]
22. Livshits, V.A.; Zakataeva, N.P.; Aleshin, V.V.; Vitushkina, M.V. Identification and characterization of the new gene rhtA involved in threonine and homoserine efflux in Escherichia coli. *Res. Microbiol.* **2003**, *154*, 123–135. [CrossRef]
23. Lee, J.H.; Sung, B.H.; Kim, M.S.; Blattner, F.R.; Yoon, B.H.; Kim, J.H.; Kim, S.C. Metabolic engineering of a reduced-genome strain of Escherichia coli for L-threonine production. *Microb. Cell Factories* **2009**, *8*, 2. [CrossRef] [PubMed]
24. Lee, J.H.; Jung, S.C.; Bui, L.M.; Kang, K.H.; Song, J.J.; Kim, S.C. Improved production of L-threonine in Escherichia coli by use of a DNA scaffold system. *Appl. Environ. Microbiol.* **2013**, *79*, 774–782. [CrossRef] [PubMed]
25. Zhao, L.; Zhang, H.; Wang, X.; Han, G.; Ma, W.; Hu, X.; Li, Y. Transcriptomic analysis of an l-threonine-producing Escherichia coli TWF001. *Biotechnol. Appl. Biochem.* **2020**, *67*, 414–429. [PubMed]
26. Reinscheid, D.J.; Kronemeyer, W.; Eggeling, L.; Eikmanns, B.J.; Sahm, H. Stable Expression of hom-1-thrB in Corynebacterium glutamicum and Its Effect on the Carbon Flux to Threonine and Related Amino Acids. *Appl. Environ. Microbiol.* **1994**, *60*, 126–132. [CrossRef] [PubMed]
27. Van Dyk, T.K. Bacterial efflux transport in biotechnology. *Adv. Appl. Microbiol.* **2008**, *63*, 231–247.
28. Xu, S.; Zhang, L.; Zhou, S.; Deng, Y. Biosensor-Based Multigene Pathway Optimization for Enhancing the Production of Glycolate. *Appl. Environ. Microbiol.* **2021**, *87*, e0011321. [CrossRef]
29. Mazumder, M.; McMillen, D.R. Design and characterization of a dual-mode promoter with activation and repression capability for tuning gene expression in yeast. *Nucleic Acids Res.* **2014**, *42*, 9514–9522. [CrossRef]
30. Liu, Y.; Li, Q.; Zheng, P.; Zhang, Z.; Liu, Y.; Sun, C.; Cao, G.; Zhou, W.; Wang, X.; Zhang, D.; et al. Developing a high-throughput screening method for threonine overproduction based on an artificial promoter. *Microb. Cell Factories* **2015**, *14*, 121. [CrossRef]

31. Tan, S.Z.; Prather, K.L. Dynamic pathway regulation: Recent advances and methods of construction. *Curr. Opin. Chem. Biol.* **2017**, *41*, 28–35. [CrossRef]
32. Zhou, S.; Yuan, S.F.; Nair, P.H.; Alper, H.S.; Deng, Y.; Zhou, J. Development of a growth coupled and multi-layered dynamic regulation network balancing malonyl-CoA node to enhance (2S)-naringenin biosynthesis in Escherichia coli. *Metab. Eng.* **2021**, *67*, 41–52. [CrossRef]
33. Zhang, Y.; Wei, M.; Zhao, G.; Zhang, W.; Li, Y.; Lin, B.; Li, Y.; Xu, Q.; Chen, N.; Zhang, C. High-level production of l-homoserine using a non-induced, non-auxotrophic Escherichia coli chassis through metabolic engineering. *Bioresour. Technol.* **2021**, *327*, 124814. [CrossRef]
34. Liang, C.; Zhang, X.; Wu, J.; Mu, S.; Wu, Z.; Jin, J.M.; Tang, S.Y. Dynamic control of toxic natural product biosynthesis by an artificial regulatory circuit. *Metab. Eng.* **2020**, *57*, 239–246. [CrossRef]
35. Zakataeva, N.P.; Aleshin, V.V.; Tokmakova, I.L.; Troshin, P.V.; Livshits, V.A. The novel transmembrane Escherichia coli proteins involved in the amino acid efflux. *FEBS Lett.* **1999**, *452*, 228–232. [CrossRef]
36. Li, Y.; Wei, H.; Wang, T.; Xu, Q.; Zhang, C.; Fan, X.; Ma, Q.; Chen, N.; Xie, X. Current status on metabolic engineering for the production of l-aspartate family amino acids and derivatives. *Bioresour. Technol.* **2017**, *245*, 1588–1602. [CrossRef]
37. Lerner, C.G.; Inouye, M. Low copy number plasmids for regulated low-level expression of cloned genes in Escherichia coli with blue/white insert screening capability. *Nucleic Acids Res.* **1990**, *18*, 4631. [CrossRef]